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Cloning, Expression, Purification, and Analysis of Mannitol Dehydrogenase Gene *mtlK* from *Lactobacillus brevis*

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Abstract

The commercial production of mannitol involves high-pressure hydrogenation of fructose using a nickel catalyst, a costly process. Mannitol can be produced through fermentation by microorganisms. Currently, a few *Lactobacillus* strains are used to develop an efficient process for mannitol bioproduction; most of the strains produce mannitol from fructose with other products. An approach toward improving this process would be to genetically engineer *Lactobacillus* strains to increase fructose-to-mannitol conversion with decreased production of other products. We cloned the gene *mtlK* encoding mannitol-2-dehydrogenase (EC 1.1.1.67) that catalyzes the conversion of fructose into mannitol from *Lactobacillus brevis* using genomic polymerase chain reaction. The *mtlK* clone contains 1328 bp of DNA sequence including a 1002-bp open reading frame that consisted of 333 amino acids with a predicted molecular mass of about 36 kDa. The functional mannitol-2-dehydrogenase was produced by overexpressing *mtlK* via pRSETa vector in *Escherichia coli* BL21pLysS on isopropyl-β-D-thiogalactopyranoside induction. The fusion protein is able to catalyze the reduction of fructose to mannitol at pH 5.35. Similar rates of catalytic reduction were observed using either the NADH or NADPH as cofactor under in vitro assay conditions. Genetically engineered *Lactobacillus plantarum* TF103 carrying the *mtlK* gene of *L. brevis* indicated increased mannitol production from glucose. The evaluation of mixed sugar fermentation and mannitol production by this strain is in progress.

Index Entries: Lactic acid bacteria; mannitol-2-dehydrogenase; NAD(P)H; *mtlK*; mannitol fermentation; *Lactobacillus*.

Introduction

Mannitol is a six-carbon polyalcohol that is widely distributed in nature, where it functions as an osmolyte capable of protecting cells

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†Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the names by USDA implies no approval of the product to the exclusion of others that may also be suitable.

against various forms of stress. D-Mannitol has a sweet taste with lower calories compared with six-carbon sugars and is an ideal sugar substitute in food, medicine, and pharmaceutical products. The commercial production of mannitol involves high-pressure hydrogenation of fructose using a nickel catalyst, a fairly costly and inefficient process *(1,2)*. The fact that mannitol can be produced through fermentation processes by microorganisms provides alternative means of production and, therefore, has received much attention.

Microorganisms such as the fungi *Aspergillus candidus (3)* and *Penicillum* sp. *(4)*, the yeasts *Torulopsis mannitofaciens (5,6)* and *Candida magnoliae (7,8)*, and the bacterial species *Acetobacter suboxydans (9)*, *Agaricus campestris (10)*, and certain lactic acid bacteria (LAB) have been described to produce mannitol from various carbon sources. Among these microorganisms, the heterofermentive LAB strains *Lactobacillus sanfranciscensis (11)*, *Leuconostoc mesenteroides (12)*, and *Lactobacillus fermentum (13,14)* have been reported to produce mannitol from fructose with high yield and efficiency *(15)*.

LAB use different strategies to synthesize and utilize D-mannitol. Homofermentative LAB such as *Lactobacillus plantarum* strains use a sugar phosphotransferase transport system and mannitol-1-phosphate 5-dehydrogenase (EC 1.1.1.17, encoded by *mtlD*) to assimilate D-mannitol from the environment and convert it via D-mannitol-1-phosphate into D-fructose-6-phosphate when glucose is unavailable. The fructose-6-phosphate is then catabolized through glycolysis.

Heterofermentative LAB such as *Lactobacillus brevis* and *Lactobacillus. fermentum* oxidize D-mannitol directly to D-fructose by mannitol-2-dehydrogenase (EC 1.1.1.67, encoded by *mtlK*), and the reversible reaction catalyzed by mannitol-2-dehydrogenase is

$$\text{D-mannitol} + NAD^+ \leftrightarrow \text{D-fructose} + NADH, H^+$$

Current mannitol-producing heterofermentative LAB strains produce mannitol solely from fructose via the reverse reaction that is carried out at lower pH (5.0–5.5) with higher D-fructose concentration. Mannitol-2-dehydrogenase is the key enzyme for mannitol production in mannitol-producing LAB strains. The purification of mannitol-2-dehydrogenase from *L. brevis* was described earlier *(16)*, but the gene coding for this enzyme has not yet been cloned. The specific mannitol dehydrogenase gene from *L. brevis* needs to be studied before advanced genetic manipulations for the use of mixed sugars for mannitol production can take place. Here we describe our work in molecular cloning and characterization of the *mtlK* gene from *L. brevis* with preliminary data on engineered LAB for mannitol production.

Materials and Methods

Bacterial Strains and Growth Conditions

L. brevis strain ATCC 367 was maintained on MRS plates and grown in liquid medium without shaking at 30°C. *Escherichia coli* strain *Dh5α* and BL21(DE3)pLysS cells were grown at 37°C in Luria-Bertani medium supplemented with 100 μg/mL of ampicillin and/or 35 μg/mL of chloramphenicol when necessary. *L. plantarum* NCIMB8826 derivative strain TF103 was provided by Dr. Ferain *(17)*. This strain, defective for both D- and L-lactate dehydrogenase activities, was grown in MRS broth with chloramphenicol (10 μg/mL) at 110 rpm and 37°C. Plasmid pTRKH$_2$ was kindly provided by Dr. Klaenhammer *(18)* and maintained in *Dh5α* cells with BHI medium containing erythromycin (150 μg/mL).

Polymerase Chain Reaction Cloning and DNA Manipulation

Chromosomal DNA from *L. brevis* strain ATCC 367 was prepared using a Bactozol Kit (Molecular Research Center, Cincinnati, OH) as described in the manufacturer's protocol. This genomic DNA was used as template for polymerase chain reaction (PCR) using primer pairs BremtlK5′ 330 and BremtlK3′ 1657, which were designed from the partially assembled *L. brevis* database (http://genome.jgi-psf.org/draft_microbes/lacbr/lacbr.home.html). The *mtlK*-specific sequence was amplified and cloned into pTA vector (Invitrogen, Carlsbad, CA). Plasmid DNA manipulations in *E. coli* were performed as described previously *(19)*, and all the constructs were confirmed by sequencing by the ABI Prism 310 using an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA). Sequence analyses were performed using the SDSC Biology WorkBench (http://www.sdsc.edu/Research/biology/) and NCBI (http://www.ncbi.nlm.nih.gov/).

Overexpression of mtlK *in* E. coli

The full-length open reading frame (ORF) of *mtlK* was subcloned into pRSETa (Invitrogen) to overexpress the protein and assess the mannitol dehydrogenase activity in *E. coli* BL21pLysS cells. A *Bam*HI site was introduced using BremtlKBamATG5′ (Table 1) in front of the ATG start codon so that the recombinant protein was in frame with the His tag sequences from the vector, and a *Kpn*I site was introduced into BremtlKKpn3′ (Table 1). The *Bam*HI- and *Kpn*I-digested PCR products were cloned into the *Bam*HI and *Kpn*I site of pRSETa to obtain pRSETmtlK, which was introduced into BL21pLysS. The isopropyl-β-D-thiogalactopyranoside (IPTG)- induced fusion protein expression of the pRSETmtlK recombinant BL21pLysS was performed according to the manufacturer's instructions (Invitrogen).

Table 1
Oligonucleotides Used and Their Sequences*

Oligonucleotide	Sequence
BremtlKBamATG5′	CCA**GGATCC**ATGAAAGCTTTAGT
BremtlKKpn3′	ACT**GGTACC**TTCACAACTGCCTTGCTGA
BremtlK5′330	GGTGAGTGAAGCCTTTCTGG
BremtlK3′1657	GCCGAATTAATCACAAGGGA
M13 reverse	CAGGAAACAGCTATGAC
T7 promoter	TAATACGACTCACTATAGGG
H_2 5′ primer	AACAGCTATGACCA
H_2 3′ primer	GTAAAACGACGGCCAGT

*Bold nucleotides indicate the restriction enzyme sites that were introduced into the primer.

Purification of Fusion Protein in E. coli

A fresh overnight culture of the recombinant pRSETmtlK BL21pLysS was used to inoculate 30 mL of SOB medium (20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 186 mg of KCl, all at pH 7.0) containing ampicillin (50 μg/mL) and chloramphenicol (35 μg/mL). IPTG (final concentration: 1 m*M*) was added to the culture once the OD_{600} reached about 0.4–0.5. The cells were grown for an additional 5–7 h. The cell pellet was lysed with BugBuster reagent and the lysate was purified using a HisBind purification kit (Novagen, Madison, WI) following the manufacturer's instructions. Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). Samples (10 μL, about 1-10 μg) of eluted fractions were used for electrophoresis on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Mannitol-2-dehydrogenase Assay

Mannitol-2-dehydrogenase activity was assayed by measuring the rate of oxidation of NAD(P)H using fructose as substrate as described previously *(16,20)* with modifications. A reaction mixture containing 1 mL of 20 m*M* sodium phosphate buffer (pH 5.35), 0.1 m*M* NAD(P)H, and sufficient diluted cell lysate was first incubated for 2 min at 30°C; then fructose was added to a final concentration of 100 m*M*. Enzyme activity is based on the decrease in OD_{340} compared to the absorbance without fructose and expressed as micromoles of NAD(P)H oxidized per minute per milligram of protein.

Genetic Engineering of L. plantarum TF103

The *mtlK* gene in pTA vector was digested with *Xba*I and *Sst*I and cloned into the *Xba*I and *Sst*I sites of $pTRKH_2$ shuttle vector. The resultant $pTRKH_2$mtlK was confirmed by sequencing using vector-specific primers (H_2 5′ primer and H_2 3′ primer, Table 1). *L. plantarum* TF103 transformation

was performed as described previously *(13)*. Briefly, a 48-h culture from a single colony on a fresh MRS chloramphenicol (10 μg/mL) plate was inoculated in MRS at 2% with 10 μg/mL of chloramphenicol and 1% glycine and grown at 120 rpm and 37°C until reaching an OD_{600} of 0.5. The cells were washed twice with ice-cold washing buffer (5 m*M* $NaPO_4$, pH 7.4; and 1 m*M* $MgCl_2$) and resuspended to 1% of the original culture volume with ice-cold electroporation buffer (10% glycerol, 0.9% sucrose, 3 m*M* $MgCl_2$). Forty-five microliters of cells was mixed with 1 to 2 μL of plasmid DNA (100–500 ng) in a 0.2-cm cuvet and electroporated using a MicroPulser (Bio-Rad) set to 3.0 kV, 4.0 ms. Immediately after the pulsing, the cells were kept on ice for 10 min, then incubated at 37°C for 1 to 2 h before plating on MRS with erythromycin (5 μg/mL).

High-Performance Liquid Chromatography Analysis for Mannitol Production

Flask fermentations were conducted using recombinant *L. plantarum* TF103 carrying $pTRKH_2$mtlK in MRS liquid with an additional 2% glucose as described previously *(21)*. Briefly, single colonies of the recombinant and parent TF103 were inoculated into 3 mL of MRS broth with chloramphenicol alone or chloramphenicol and erythromycin together and incubated overnight at 37°C with shaking at 110 rpm. The cells were collected and seeded at an OD_{600} of 0.5 into 20 mL of MRS with corresponding antibiotics and an additional 2% glucose in 50-mL flasks capped with rubber stoppers vented with 22-gage needles for fermentation. The concentration of glucose and mannitol were measured after 120 h by high-performance liquid chromatography (HPLC) on an Aminex HPX-87P column as described *(14)*.

Results and Discussion

A Blast search using *mtlK* (AY090766) of *L. mesenteroides (22)* indicated that the *mtlK* gene of *L. brevis* is located at scaffold17 between 14465 and 13464 of the draft *L. brevis* genome. A total of 1327 bp of *mtlK* gene was obtained through genomic PCR of *L. brevis* ATCC 367 using primers designed from scaffold17. The sequence included 185 bp in front of the ATG start codon with 5′ promoter sequences, 1002 bp ORF (333 amino acids), and 140 bp of 3′ flanking sequences after the TAG stop codon. The coding sequence has a predicted molecular mass of 36 kDa. A putative ribosome-binding site (AGGA) is located 14 bp upstream of the ATG start codon.

Multiple sequence alignments (Fig. 1) indicated that the *mtlK* gene contains the conserved G33 that was suggested as a key residue for cofactor binding in all members of the dehydrogenase superfamily *(23,24)*. Another cofactor NAD(P)H fingerprint region GDGFMG, often found at the N-terminus of most dehydrogenases, is located at the inter domain of the MtlK. This appears to be an unusual characteristic of a new family of mannitol dehydrogenases, which belongs to the medium-chain ehydrogenase/

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1   MEALVLTGTK KLEVENIEQP EVKPNEVLIH TAFAGICGTD HALYAGLPGS
2   MEALVLTGTK KLEVKDIDRP KVLPNEVLIH TAFAGICGTD HALYAGLPGS
3   MKALVLTGTK QFEMQDVTTP TVKDDEVLVN TAYAGICGTD RALYAGLPGS

1   ADAVPPIVLG HENSGVVAEI GSDVTNVAVG DRVTIDPNIY CGQCKYCRTA
2   ADAVPPIVLG HENSGVVAEI GSAVTNVKVG DRVTVDPNIY CGQCKYCRTA
3   ADAVPPIVLG HENSGIVAAI GRNVTNVKVG DRVTVDPNIY CGECEYCRTD

1   RPELCENLSA VGVTRNGGFE EYFTAPASVV YQIPDNVSLK SAAVVEPISC
2   RPELCENLSA VGVTRDGGFE EFFTAPASVV YPIPDNVSLK SAAVVEPISC
3   RPELCDNLSA VGVTRDGGLE ESFTAPASVV YPIPDSVSLK AAATVEPISC

1   AVHGIQLLKV TPYQKALVIG DGFMGELFVQ ILQAYGIHQV DLAGIVPEKL
2   AVHGIQLLKV TPYQKALVIG DGFMGELFVQ ILQAYGIHQV DLAGIVDEKL
3   AVHGVKLLDL TPYQKALVIG DGFMGQLFVQ LLQAYGVHQV DFAGIVDEKL

1   AMNKEKFGVK NTYNTKDGDK IPEGTYDVVV EAVGLPQTQE AAIEASARGA
2   AMNKEKFGVK NTYNTMKGDK IPEGEYDVII EAVGLPQTQE AAIEASARGA
3   AFNKEKFGVT NTYNTTR-DS IP-ADYDVVI EAVGLPQTQE QAVEATKKGA

1   QVLMFGVGGP DAKFQMNTYE VFQKQLTIQG SFINPNAFED SLALLSSGKL
2   QVLMFGVGGP DAKFQMNTYE VFQKQLTIQG SFINPNAFED SLALLSSGKL
3   QVLMFGVGKP NQTFSMNTYE VYQKQLKIQG AFINPYAFED SIALLASGQL

1   DVESLMSHEL DYQTVDDFVN GKLGVVSKAV VKVGGEEA
2   NVEALMSHEL DYKTVDDFVN GKLGVVSKAV VKVGGEEA
3   DVESLISHEV SLEQVEDVLN GKVEHVSKAV VKVSD---
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Fig. 1. Comparison of amino acid sequences of three different *mtlK*-encoded mannitol-2-dehydrogenases from *L. pseudomesenteroides* (1), *L. mesenteroides* (2), and. *L. brevis* (3). ***G*** is conserved in the N-terminal coenzyme-binding domain; ***D*** and ***K*** are conserved residues for catalytic active sites, and **GDGFMG** is the conserved cofactor NAD(P)H-binding domain. ***C*** (91, 94, 97 and 105) and ***E*** (146) are conserved residues for a zinc-binding structure.

reductase (MDR) subfamily *(22)*. The C-terminal domain of MtlK contains the Asp 230–Lys 295 region for enzyme-active sites as proposed for mannitol binding *(23)*. Four cysteine residues (C 91, 94, 97, and 105) and one glutamine (E 146) residue present in MtlK are conserved within all zinc-containing alcohol dehydrogenases *(22)* and may contribute to a zinc-binding structure that is responsible for zinc-dependent enzyme activity.

The *mtlK* gene from *L. brevis* possesses 75.4 and 74.6% identity with two recently isolated clones from *L. mesenteroides (22)* and *Leuconostoc pseudomesenteroides (25)*, and little similarity with other known microbial mannitol dehydrogenase genes (Table 2). Dot blot hybridization signals appeared strongly in *L. mesenteroides* and *L. pseudomesenteroides* and weakly in *L. brevis* and *L. fermentum* when *L. mesenteroides mdh* (*mtlK*) gene probe was used to hybridize with nine different bacteria chromosomal DNA including seven LAB, *E. coli*, and *Pseudomonas fluorescens (22)*. These results suggest that the mannitol-2-dehydrogenases from *L. mesenteroides*,

Table 2
Percentage Identities of Amino Acid Sequences from Available Mannitol Dehydrogenases in LAB Strains

	L. brevis (%)	*L. mesenteroides* (%)	*L. pseudomesenteroides* (%)	*L. lactis* (%)	*L. plantarum* (%)
L. brevis mtlK	100	75.4	74.6	10.1	10.3
L. plantarum mtlD	10.3	11.2	11.2	51.4	100

L. pseudomesenteroides, and *L. brevis* can be classified as new members of the MDR family of mannitol dehydrogenase. Similar enzymes from *L. fermentum (20)* and *L. sanfranciscensis (26)* might also belong to this group, although the genes have not been cloned yet.

The *mtlK* gene was induced by IPTG in recombinant *E. coli*. The fusion protein contains an additional 36 amino acids including six His residues at the N-terminal from the vector, resulting in an addition of about 3 kDa of the total protein mass. A band corresponding to a molecular mass of about 46 kDa, instead of the predicted 39 kDa (36 + 3), was detected on SDS-PAGE gel after Ni-column purification (Fig. 2). The abnormal, slower than predicted migration on SDS-PAGE was also observed in *L. mesenteroides* (41 kDa for the predicted 36 kDa) *(22)* and *L. pseudomesenteroides* (43 kDa for the predicted 36 kDa) *(25)*. This divergence may be owing to the low pI (the predicted pI for *L. brevis* MtlK is 4.42) of proteins in this novel subfamily of mannitol dehydrogenase with unique amino acid sequences that result in high negative charge (–20) at pH 8.0 *(25)*.

In vitro enzyme assays indicated that MtlK can use both NADH and NADPH as cofactor (Fig. 3) for fructose reduction at pH 5.35. The rates of fructose catalytic reduction appeared similar with either NADH or NADPH as cofactor. This is unusual for a prokaryotic mannitol dehydrogenase, because, except for in *L. fermentum* and *L. sanfranciscensis*, most of the enzymes are NADH dependent, whereas in eukaryotic fungi and yeasts, the dehydrogenases are NADPH dependent *(20,24,26)*. Similar to the dehydrogenases *(20,25,26)* in this new subfamily, *L. brevis* mannitol-2-dehydrogenase activities were zinc dependent (data not shown).

Flask fermentations under aerobic conditions of recombinant *L. plantarum* TF103 indicated that about 17 m*M* mannitol was produced from MRS medium with additional 2% glucose (Table 3). The TF103 strain lacking functional *l-ldh* and *D-ldh* for lactate dehydrogenases was reported to produce a trace amount of mannitol (4 m*M*) from glucose for regeneration of NAD^+ *(27)*. *L. plantarum* is homofermentive and does not have *mtlK* gene. Our preliminary result indicated that the *mtlK*-engineered strain of this organism was able to produce fourfold greater mannitol from glucose compared with the reported mannitol production in TF103 *(27)*. Mannitol was

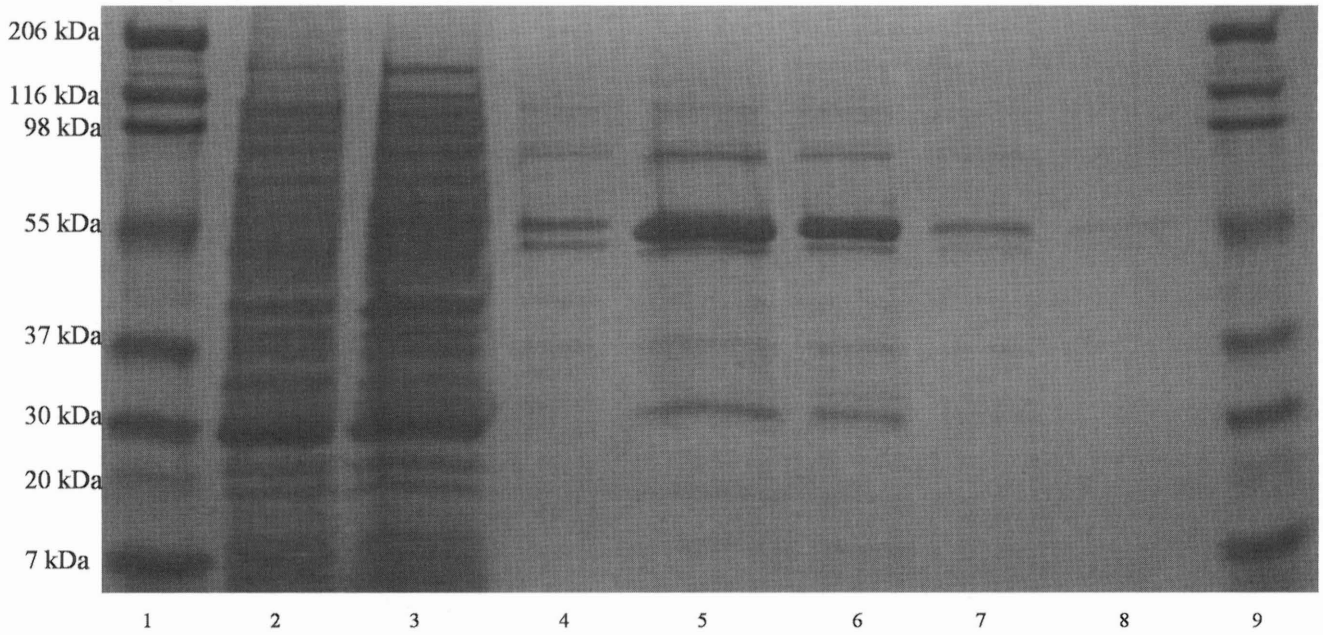

Fig. 2. IPTG-induced overexpression of *mtlK* gene and purification of *L. brevis* mannitol-2-dehydrogenase in recombinant *E. coli* BL21(DE3) pLysS cells. Lanes 1 and 9, Bio-Rad SDS-PAGE broad range prestained standards; lane 2; cell lysate from BL21pLysS transformed with pRSETmtlK-IPTG; lane 3, BL21pLysS pRSETmtlK + IPTG; lanes 4–8; fractions from Ni column purification of His-tagged MtlK.

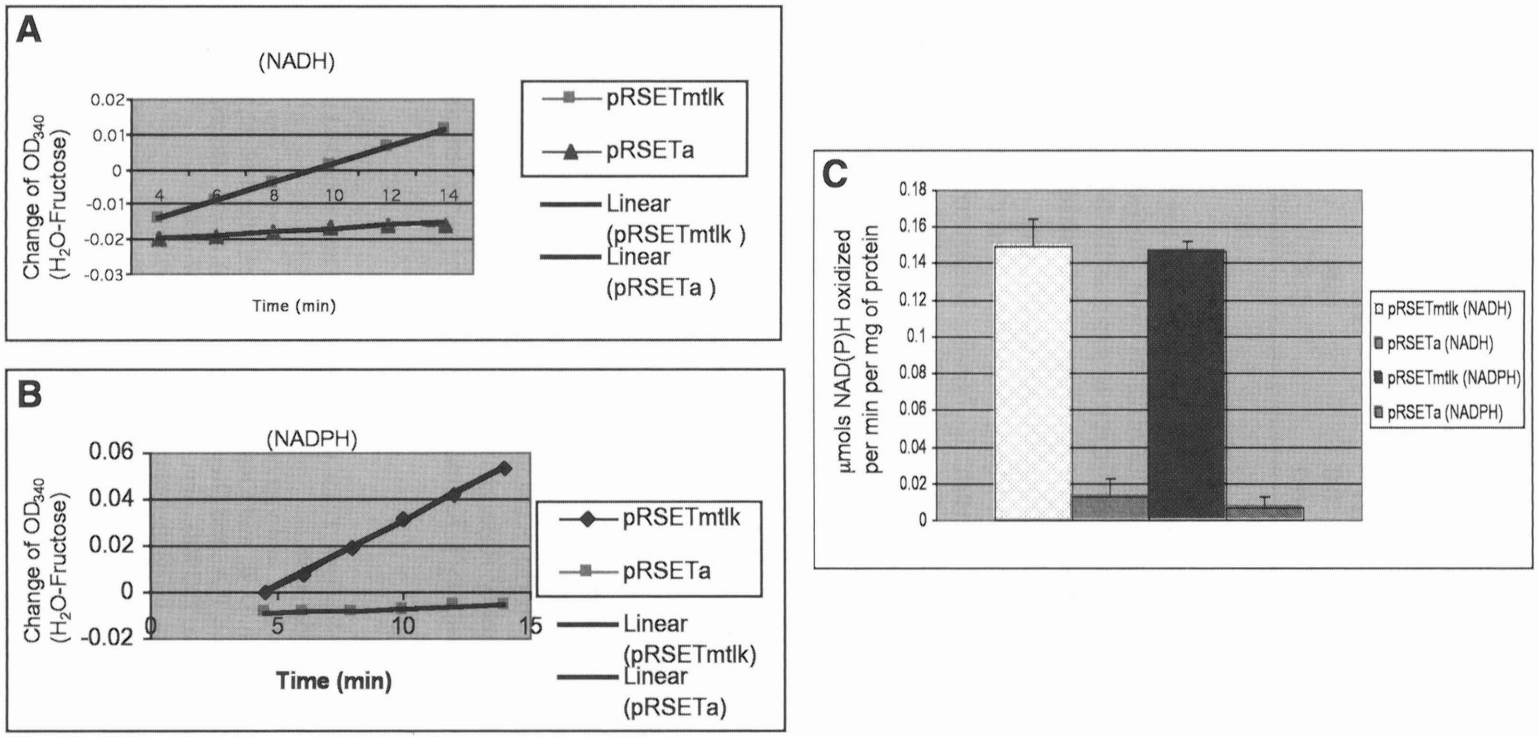

Fig. 3. (A) Rate of fructose reduction using NADH as cofactor by overexpressed Mtlk. **(B)** Rate of fructose reduction using NADH as cofactor by overexpressed Mtlk. **(C)** MtlK enzymatic activities from cell lysate of pRSETmtlK recombinant *E. coli* BL21pLysS using fructose as substrate and NADH and NADPH as cofactors at pH 5.35. Enzyme activities were measured from three independent assays.

Table 3
HPLC Analysis of Mannitol Production from *L. plantarum* TF103 Transformed with pTRKH$_2$mtlK

	TF103	TF103 *mtlK*-1	TF103 *mtlK*-2
Glucose consumed (m*M*)	221	217	218
Mannitol produced (m*M*)	0.0	16.8	17.4
% g Mannitol/g glucose	0.0	7.8	8.0

undetectable in our HPLC assay in TF103 (Table 3). This result suggests that the cloned *mtlK* from *L. brevis* containing its own promoter (185 bp) and 3′ flanking sequences (140 bp) is sufficient to express the MtlK in *L. plantarum* TF103. Additional comprehensive analyses of this strain for mixed sugar fermentation and mannitol production are under way.

In summary, we have identified, amplified, and cloned the mannitol dehydrogenase gene *mtlK* from *L. brevis*. Mannitol-2-dehydrogenase belongs to a new subfamily of the medium-chain alcohol/polyol dehydrogenase/reductase protein family (MDR). The *mtlK* recombinant *E. coli* produced His-tagged MtlK on IPTG induction, and the fusion protein was able to catalyze the reduction of mannitol from fructose at pH 5.35. This enzyme can use either NADH or NADPH as cofactor for mannitol production. Work on manipulating LAB utilizing mixed sugars as substrate to produce mannitol with high yield and less byproducts is in progress.

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